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Molecular cloning and enzymatic characterization of a *Trichoderma reesei* 1,2-α-D-mannosidase

Marleen Maras [a], Nico Callewaert [a], Kathleen Piens [b], Marc Claeyssens [b], Wim Martinet [a], Sylviane Dewaele [a], Hans Contreras [a], Isabelle Dewerte [a], Merja Penttilä [c], Roland Contreras [a,*]

[a] *Department of Molecular Biology, Flanders Interuniversity Institute for Biotechnology and University of Ghent, K.L. Ledeganckstraat 35, 9000 Ghent, Belgium*

[b] *Department of Biochemistry, Physiology and Microbiology, University of Ghent, K.L. Ledeganckstraat 35, 9000 Ghent, Belgium*

[c] *VTT Biotechnology and Food Research, Biologinkuja 1, 02044 Espoo, Finland*

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Abstract

A cDNA encoding 1,2-α-D-mannosidase *mds1* from *Trichoderma reesei* was cloned. The largest open reading frame occupied 1571 bp. The predicted sequence contains 523 amino acid residues for a calculated molecular mass of 56 266 Da and shows high similarity to the amino acid sequences of 1,2-α-D-mannosidases from *Aspergillus saitoi* and *Penicillium citrinum* (51.6 and 51.0% identity, respectively). *T. reesei* mannosidase was produced as a recombinant enzyme in the yeast *Pichia pastoris*. Replacement of the N-terminal part with the prepro-signal peptide of the *Saccharomyces cerevisiae* α-mating factor resulted in high amounts of secreted enzyme. A three-step purification protocol was designed and the enzymatic properties were analysed. The enzyme was characterized as a class-I mannosidase.

Keywords: Mannosidase; Oligosaccharide; Yeast; *Trichoderma reesei*; Glycosidase; Fungus

1. Introduction

N-Linked oligosaccharide synthesis has been studied extensively both in mammalian cells and in the yeast *Saccharomyces cerevisiae* (Rademacher et al., 1988; Herscovics and Orlean, 1993). The knowledge gained has often been used to predict or explain glycosylation events in other less well-studied organisms, such as filamentous fungi. In the latter case, however, many questions remain unanswered, especially those concerning the role and location of some fungal glycosidases and glycosyltransferases. It has been observed that *N*-glycans synthesized by filamentous fungi are smaller and simpler, and often more resemble mammalian high-mannose *N*-glycans than the oligosaccharides synthesized by *S. cerevisiae* (Palamarczyk et al., 1998). This is one of the

* Corresponding author. Fax: +32-9-264-8798.

E-mail address: roland.contreras@dmb.rug.ac.be (R. Contreras)

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reasons why filamentous fungi are attractive host organisms for the production of mammalian glycoproteins with therapeutic value. Recently, efforts have been made to elucidate their glycosylation pathway. It is observed that a common $Glc_3Man_9GlcNAc_2$ precursor oligosaccharide is synthesized in most eukaryotes and transferred to nascent proteins. In filamentous fungi, structural elucidations suggest trimming by glucosidases and mannosidase(s) to glycans as small as $Man_5GlcNAc_2$. This knowledge has been used to convert fungal glycans to mammalian hybrid structures (Maras et al., 1997b).

Aspergillus and *Penicillium* 1,2-α-D-mannosidases have been characterized and their genes cloned (Yamashita et al., 1980; Yoshida et al., 1993; Inoue et al., 1995; Yoshida and Ichishima, 1995). The presence of conserved regions that are common among a growing list of higher eukaryotic genes, suggests that they belong to a gene family known as class-I mannosidases (Moremen et al., 1994). Class-I enzymes share common enzymatic characteristics. They only cleave α-1,2-linked D-mannoses, but not arylglycosides, and they are inhibited by deoxymannojirimycin, but not by swainsonine. Different pH and temperature optima, subcellular locations, as well as K_m and K_i values were found. In this paper, we present the cDNA sequence of a 1,2-α-D-mannosidase from the filamentous fungus *Trichoderma reesei*. The mannosidase was efficiently produced in the heterologous host *Pichia pastoris*. Enzymatic characterization demonstrates that the mannosidase is closely related to those from *A. saitoi* and *P. citrinum*. However, some differences are discussed also.

2. Materials and methods

2.1. Products and plasmids

Superscript reverse transcriptase was purchased from Gibco BRL (Paisley, Scotland). Plasmids pGEM-T and pGEM4 were purchased from Promega (Madison, WI). A random-primed labelling kit was purchased from Boehringer Mannheim (Mannheim, Germany). *P. pastoris* strain GS115 and plasmids pPIC9 and pPICZ(B) were obtained from Invitrogen (San Diego, CA). All columns and resins for enzyme purification were purchased from Pharmacia Biotech (Uppsala, Sweden). Man-α-1,2-D-Man, Man-α-1,3-D-Man and Man-α-1,6-D-Man were obtained from Dextra Laboratories (Reading, UK). $Man_8GlcNAc$ was from BioCarb Chemicals (Lund, Sweden). Swainsonine, deoxymannojirimycin and reference $Man_{(5-9)}GlcNAc_2$ oligosaccharides were obtained from Oxford Glycosystems (Oxford, UK). Glycopeptidase F (PNGase F) was obtained from Biolabs (Beverly, MA).

2.2. Isolation of the 1,2-α-D-mannosidase probe

cDNA was synthesized from *T. reesei* mRNA by Superscript reverse transcriptase using a degenerate oligonucleotide 5′-$T_{/C}T_{/C}AIAA_{/G}C_{/T}TCG_{/A}$ $TCG_{/A}TG$-3′ as a primer. This single-stranded cDNA served as template to amplify a fragment of the mannosidase gene by PCR, using 1.6 μM of a set of degenerate primers 5′-CGC AAG CTT $A_{/G}TG$ $A_{/G/C/T}GC$ $C_{/T}TC$ $A_{/G/C/T}GT$ $A_{/G}TT$ $A_{/G}AA$-3′ and 5′-CGC GAA TTC $GAC_{/T}$ TCI $TTC_{/T}$ $TAC_{/T}$ $GAA_{/G}$ $TAC_{/T}$ $C_{/T}TI$ $T_{/C}TA_{/G/C/T}$ AA-3′ (Herscovics et al., 1994). PCR reactions were conducted with 2.5 mU of Taq polymerase, using the following programme: 1 min at 95°C, 5 min at 80°C (addition of polymerase), 35 cycles of 1 min at 95°C, 1 min at 55°C, 3 min at 72°C and finally a 3-min extension step at 72°C. The obtained 750-bp PCR product was cloned in a pGEM-T vector and sequenced following an ABI Taq DyeDeoxy terminator cycle sequencing protocol (ABI, Foster City, CA). Samples were run on an automated ABI373A sequencing system (ABI). The mannosidase fragment was recovered from pGEM-T by a *Sph*I/*Nsi*I double digest and further used as probe to screen a *T. reesei* cDNA library.

2.3. Screening of a T. reesei cDNA library

A cDNA bank (Margolles-Clark et al., 1996) was prepared from *T. reesei* QM9414 grown in medium (Maras et al., 1997b) containing cellu-

lose, spent grain, glucomannan and lactose. cDNA was made with a ZAP cDNA synthesis kit (Stratagene Cloning Systems, La Jolla, CA) and ligated into an *Eco*RI/*Xho*I-cut plasmid pAJ401. The latter was derived from pFL60 (Minet and Lacroute, 1990) in which *Eco*RI and *Xho*I sites had switched orientations. The cDNA bank was transformed to *Escherichia coli* MC1061. Individual clones were screened using the mannosidase fragment probe which had been labelled by random-primed labelling. The cDNA of a positive clone was sequenced. After *Eco*RI/*Xho*I digest, the gene was cut into two smaller fragments, which were subcloned in pUC19. With the plasmids obtained, single digests with *Kpn*I or *Hin*dII or *Sph*I or *Hin*dIII cut the *mds1* gene at different positions within the coding sequence. Upon self-ligation of the plasmid, sequencing of smaller gene fragments was carried out in order to reveal the sequence of the full open reading frame.

2.4. Construction of expression vectors

The intact mannosidase gene was isolated from a pAJ401 cDNA bank vector by *Bgl*II digest, after which it was subcloned in the unique *Bam*HI restriction site of pGEM4. The resulting plasmid was named pGEM4mds1. The intact mannosidase gene was recovered after partial *Pvu*I and *Eco*RI digestion of the latter plasmid and ligated between the blunted *Bam*HI and *Eco*RI sites of pPIC9. The gene was also introduced in a second *P. pastoris* expression vector after recovery of the *mds1* gene from pPIC9 by *Eco*RI/*Bgl*II double digest and ligating to compatible sites in pPICZ (Scorer et al., 1994), which resulted in the plasmid pPP3mds1.

A construct was made for enhanced secretion of *T. reesei* mannosidase. A partial *Nar*I digest of pGEM11mds1 was followed by filling-in with Klenow fragment. After cutting with *Not*I, a 1.7-kb mannosidase gene fragment was introduced in pPIC9 (Scorer et al., 1994) by ligating blunted *Nar*I in-frame to blunted *Eco*RI of the pre-pro-signal sequence of the *S. cerevisiae* α-mating factor. This plasmid was named pPP1MFmds1.

2.5. Overexpression in P. pastoris

P. pastoris strain GS115 (Romanos, 1995) was transformed by electroporation with 20 μg of both expression vectors pPP3mds1 and pPP1MFmds1. Before transformation, pPP3mds1 was linearized with *Bgl*II just before the *P. pastoris* AOX1 promoter. pPP1MFmds1 cutting with *Bgl*II resulted in the mannosidase gene, flanked at its 5′ end by the AOX1 promoter and at its 3′ end by the *his4* gene and the AOXI terminator sequence. Transformants were grown on minimal medium plates, containing 1.34% yeast nitrogen base and 1% dextrose. Several colonies were grown to A_{600} of 2–6, in minimal medium containing 1% (v/v) glycerol as carbon source. Resuspending the cells to the same density in 1% (v/v) methanol containing minimal medium induced mannosidase secretion. The induction lasted 24 h, with an extra addition of 0.5% methanol after 12 h. Extracellular proteins were analysed by PAGE and Coomassie blue staining. The enzymatic activity of 1,2-α-D-mannosidase in the extracellular medium was determined using $Man_8GlcNAc$ as substrate (see below).

2.6. Enzyme purification

All steps were performed at 4°C. *P. pastoris*-expressing prepro-mannosidase was grown in 4 l BMGY (containing 1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base and 1% glycerol) to $A_{600} = 6$ (Juge et al., 1996). Cells were resuspended for 5 days at 28°C in BMMY (i.e. BMGY with addition of 0.5% methanol every 12 h instead of glycerol). Cells were then separated from the growth medium by centrifugation (15 min at $10\,000 \times g$) and by filtration over a 0.45-μm membrane filter.

Proteins were precipitated after addition of ammonium sulphate to 90% saturation and centrifugation for 20 min at $10\,000 \times g$. The precipitate was dissolved in 50 mM of sodium acetate/acetic acid buffer (pH 4.0) and dialysed overnight against the same buffer. This solution was applied to an S-Sepharose FF XK50 column (5×12 cm^2). Elution was started with 10 bed volumes of 50

mM of sodium acetate/acetic acid buffer (pH 4.0) containing 1 M NaCl. Enzyme active fractions were pooled and dialysed against 50 mM Tris/HCl (pH 8.0). The dialysate was incubated batchwise with 5 ml of Q-Sepharose FF. The unretained mannosidase was re-applied after pH adjustment to 4.0 to a monoS (16/10) column, as described above. Pure mannosidase was stored at −20°C, after addition of 30% (v/v) glycerol as cryoprotectant.

2.7. α-Mannosidase assays

$Man_8GlcNAc$ was tested as a substrate for mannosidase in the extracellular medium of *P. pastoris* transformants and of *T. reesei* RUTC 30. Substrate was added to 20 μl of crude extracellular medium to a final concentration of 0.02 mM and incubated for several hours at 37°C. Analysis of the reaction products was achieved by fluorophore-assisted carbohydrate electrophoresis (FACE) as described previously (Maras et al., 1997a). A mixture of $Man_{(5-9)}GlcNAc_2$ oligosaccharides was also tested as substrate.

Using Man-α-1,2-D-Man as a substrate, the kinetic parameters for purified recombinant 1,2-α-D-mannosidase were determined at pH 5.0 (50 mM sodium acetate/acetic acid) at 37°C. Initial velocities were measured in 25-μl reaction mixtures containing varying substrate concentrations (between 0.18 and 6 mM) and 90 ng enzyme (0.12 nM). Reactions were stopped after 20-min incubation by adding 25 μl of 0.3 N sodium hydroxide and analysed by high-performance anion exchange chromatography-pulsed amperometric detection. Samples were taken at five different time points and applied to a Carbopac PA-10 pellicular anion-exchange column (Dionex, Sunnyvale, CA), thermostated at 40°C. Mannose was separated from unreacted substrate by using 16 mM sodium hydroxide as an eluant for 20 min. Quantification followed after calibration of the column and using Dionex Peaknet Automatic software (release 4.11). K_m and k_{cat} values were determined by non-linear fitting of the Michaelis–Menten equation using Kaleidograph 3.0.5.

S. cerevisiae invertase (Roche Molecular Biochemicals, Basel, Switzerland) was also tested as a substrate at a concentration of 7.5 mg/ml. Trimming during 10–60 min incubation with 2.4 μg mannosidase per 20 μl reaction mixture was stopped by precipitating the invertase with two volumes of ice-cold methanol. The supernatant was separated from the protein pellet and lyophilized; released mannose was measured as described previously (Mopper and Gindler, 1973).

p-Nitrophenyl-1,2-α-D-mannopyranoside (at 2-mM concentration) was tested as a substrate with 10 μg of pure recombinant *T. reesei* mannosidase or with 10 μg of jack bean mannosidase in a final volume of 200 μl (Matta and Bahl, 1972). Swainsonine was tested as inhibitor at 10 μM, 100 μM and 1 mM concentrations using Man-α-1,2-D-Man as a substrate, while inhibition by deoxymannojirimycin was assayed at concentrations ranging from 100 to 500 μM (Pan and Elbein, 1995).

2.8. PNGase F treatment

Mannosidase (12 μg) was treated with 250 U of PNGase F for 1 h at 37°C in 0.5% SDS, 1% (v/v) β-mercaptoethanol, 0.05 M Tris/HCl (pH 8.0). Incubation without PNGase F served as control.

2.9. N-terminal sequence analysis

Amino acid sequence analysis was performed by automated Edman degradation on a 477A pulsed liquid sequencer, equipped with an on-line 120A PTH analyzer (Applied Biosystems, Foster City, CA).

2.10. Computer analysis

The DNA sequence was analysed by means of a DNASIS programme. Homology searches were conducted using BLAST algorithm (Altschul et al., 1990); multiple sequence alignment was performed with CLUSTAL W algorithm (Thompson et al., 1994). Standard parameters were used in all cases. Hydropathy plots were applied as previously described (Kyte and Doolittle, 1982). A TMPRED programme was used to reveal the presence of transmembrane α-helices (Hofmann and Stoffel, 1993).

3. Results and discussion

Several filamentous fungi secrete mannosidases in their extracellular medium: specific 1,2-α-D-mannosidases are secreted by different *Aspergillus* species (Yamashita et al., 1980; Keskar et al., 1993) and by *P. citrinum* (Yoshida et al., 1993). *A. saitoi* also secretes 1,3,6-α-D-mannosidase (Amano and Kobata, 1986); an 1,2,3,6-α-D-mannosidase was found in the medium of *T. reesei* and characterized (Eneyskaya et al., 1998). Most of these enzymes are able to trim glycans that are *N*- or *O*-linked to the fungal glycoproteins. However, it is not clear whether these enzymes are also involved in intracellular processing of *N*-glycans. *T. reesei* *N*-glycans have been characterized (Maras et al., 1997a) that are not regarded to result from aspecific mannosidase trimming. To a certain extent their structures resemble the mammalian high-mannose oligosaccharides and suggest trimming by one (or possibly more) 1,2-α-D-mannosidase(s).

We cloned a *T. reesei* gene which was labelled *mds1* after heterologous expression and characterization of the gene product as a specific 1,2-α-D-mannosidase (Henrissat and Davies, 1997). It was recognized as a member of a conserved family, also referred to as class-I mannosidases (Moremen et al., 1994). The gene and the amino acid sequences show a very high degree of similarity to 1,2-α-D-mannosidases from *A. saitoi* and *P. citrinum* (Fig. 1). Using the PROTPARAM programme, a molecular mass of 56 266 Da was deduced for the 523 amino acid-containing protein. A hydropathy plot was calculated and resulted in the identification of an N-terminal hydrophobic amino acid sequence. Further analysis by the TMPRED programme suggested the presence of an 18 amino acids long transmembrane α-helix (from F3 to Y20), suggesting type-II transmembrane topology. However, helix-disrupting Pro residues are present. As can be deduced from the SIGNALP programme, it is more likely that the hydrophobic peptide is a signal sequence for secretion cleaved between Ala_{19} and Tyr_{20}. The latter hypothesis is corroborated by the presence of a $Lys_{27}Arg_{28}$ consensus cleavage site for Kex2p (Shuster, 1991).

Intact mannosidase was efficiently synthesized by *P. pastoris* and secreted to the extracellular medium. Enhancement of the secretion level was attempted by replacing the N-terminus with the prepro-sequence of the α-mating factor of *S. cerevisiae*. Ala_{25} of *T. reesei* mannosidase was fused to the prepro-signal sequence of the *S. cerevisiae* α-mating factor. Because the *Eco*RI site in plasmid pPIC9 had been used to allow in-frame cloning of the mannosidase gene fragment to the mating factor signal sequence, the peptide TyrVal-GluPhe was inserted between the mating factor signal peptide and Ala_{25} of the mannosidase. Transformants were obtained that secreted 17.5 mg of mannosidase/liter yeast culture. The amount of mannosidase secreted by the latter transformants did not seem to exceed that of *P. pastoris* transformed with pPP3mdsR. Similar amounts of mannosidase were secreted after 16 and 24 h of induction, whether secretion was directed by its own signal sequence or by that of the α-mating factor.

A three-step purification protocol was designed to obtain mannosidase of high purity, as confirmed by SDS–PAGE and Coomassie blue staining of deglycosylated mannosidase. Upon deglycosylation with PNGase F (Fig. 2), the heterogeneous mixture of enzymes was completely converted to an enzyme with an apparent molecular mass of 54 ± 2 kDa, which corresponds to the theoretical molecular mass of the secreted form of mannosidase (54 074 Da for mannosidase without its own signal peptide). After FACE (Fig. 3), the *N*-glycan mixture revealed the presence of small-sized oligosaccharides, atypical for *P. pastoris*-produced glycoproteins (Trimble et al., 1991), as well as hyperglycosylated forms.

The specific activity of pure recombinant mannosidase was determined using saturating amounts of substrate, namely 3 mM of Man-α-1,2-D-Man. One unit is defined as the amount of enzyme that releases 1 μmol mannose per minute at pH 5.0, at 37°C and under saturating conditions of substrate. A specific activity of 6.7 mU per μg of pure recombinant mannosidase was determined. With invertase from *S. cerevisiae* as a test substrate, a similar specific activity was found. To study the substrate specificity of the

Fig. 1. Alignment of amino acid sequences of 1,2-α-D-mannosidases from *T. reesei, A. saitoi, P. citrinum, S. cerevisiae* and mouse. The alignment was performed with the CLUSTAL W algorithm. Asterisks show amino acid identity; colons indicate conserved amino acid substitutions. The highest similarity was found for 1,2-α-D-mannosidase from *A. saitoi* and *P. citrinum* (51.6 and 51.0% identity, respectively).

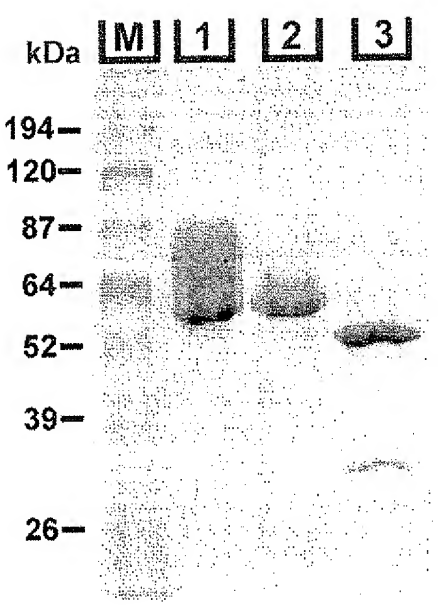

Fig. 2. Change in electrophoretic mobility of *T. reesei* 1,2-α-D-mannosidase after PNGase F treatment. 1,2-α-D-mannosidase was purified over monoS and analysed by SDS–PAGE. The purity was assessed after deglycosylation with PNGase F. Lane 1, untreated enzyme of the early eluting fractions; lane 2, untreated enzyme of the later eluting fractions; lane 3, glycanase-treated enzyme. The presence of PNGase F is detected on the gel as a weakly stained, lower migrating band. M, marker proteins.

mannosidase, different manno-oligosaccharides were tested. While Man-α-1,2-D-Man was rapidly hydrolysed to mannose, Man-α-1,3-D-Man and Man-α-1,6-D-Man were not attacked. The enzyme easily converted $Man_8GlcNAc$ or a mixture of $Man_{(6-9)}GlcNAc_2$ oligosaccharides to $Man_5GlcNAc$ and $Man_5GlcNAc_2$, respectively. Even after exhaustive digestion with 30 mU of enzyme for 3 h at 30°C, the only trimming product was $Man_5GlcNAc$ or $Man_5GlcNAc_2$. With Man-α-1,2-Man or $Man_8GlcNAc_2$ under standard assay conditions and using the same molar concentrations, approximately equal amounts of mannose were released after 20-min incubation. With *S. cerevisiae* mannan, release of mannose was readily detected by thin-layer chromatography and orcinol staining (Maras et al., 1997a). *p*-Nitrophenyl-1,2-α-D-mannopyranoside was not cleaved by *T. reesei* mannosidase, in contrast to our positive jack-bean mannosidase control.

With Man-α-1,2-D-Man as a substrate and at 37°C, an optimal pH of 5.0 was determined. Under these reaction conditions, the activity increased up to 60°C; above this temperature rapid inactivation occurred. The kinetic parameters for the same substrate were determined at pH 5.0 and at 37°C: $K_m = 600 \pm 40$ μM and $k_{cat} = 5.8 \pm 0.1$ s^{-1}. Swainsonine did not inhibit the enzyme, even at concentrations as high as 1 mM. Deoxymannojirimycin, on the other hand, was an efficient inhibitor. FACE analysis revealed that EDTA at 1 mM completely inhibited the enzyme, but this was reversed by adding Ca^{2+}.

Enzymatic characterization corroborated the high similarity to the aforementioned *Aspergillus* and *Penicillium* mannosidases with respect to pH optimum, temperature optimum and inhibition by deoxymannojirimycin. The K_m for hydrolysis of Man-α-1,2-D-Man was determined to be 600 ± 40

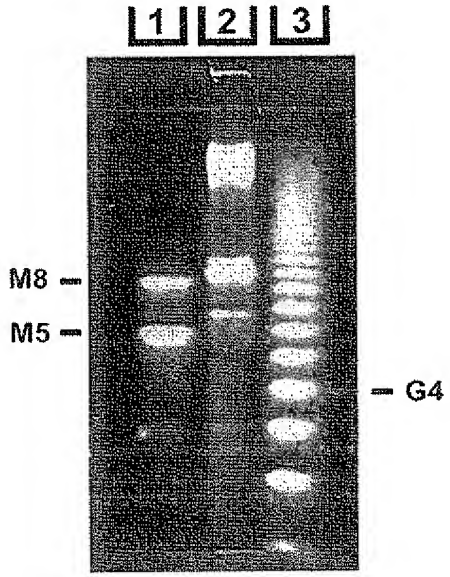

Fig. 3. FACE analysis of *N*-glycans released from *T. reesei* 1,2-α-D-mannosidase. *N*-glycans released from 40 μg mannosidase were fluorescently labelled with 8-aminonaphthalene-1,3,6-trisulphonic acid and submitted to PAGE. *N*-glycan patterns were visualized and photographed after UV excitation. The molecular mass of the mannosidase *N*-glycans (lane 2) was estimated by comparison with the position of reference $Man_5GlcNAc_2$ (M5) and $Man_8GlcNAc_2$ (M8) oligosaccharides (lane 1) or a standard oligo-Glc ladder (lane 3). G4 shows the position of the Glc tetramer.

μM for *T. reesei* mannosidase, which is similar to that of *Penicillium* mannosidase IB (Yoshida et al., 1993). The substrate specificity of this enzyme coincides with that of *A. saitoi* and *P. citrinum* mannosidases.

EDTA inhibits *T. reesei* mannosidase activity, which is restored by addition of Ca^{2+}. In the *T. reesei* sequence, however, no calcium-binding consensus sequence was identified. As already mentioned above, the presence of a cleavable signal sequence was predicted from the SIGNALP computer programme. In addition, the consensus $Lys_{27}Arg_{28}$, which is recognized by the protease Kex2p (Shuster, 1991), was identified. A Kex2p-like activity in *T. reesei* has been suggested from heterologous protein expression results (Goller et al., 1998; our unpublished results). However, it should be mentioned that cloning of the *kex2* gene of *T. reesei* has not been reported so far (Penttilä, 1998). The results suggest that this filamentous fungus secretes its mannosidase to the extracellular medium. This is in contrast with the fact that no 1,2-α-mannosidase activity was demonstrated in the medium of *T. reesei* RUTC 30 (Maras et al., 1997a). The use of different carbon sources (lactose or dextrose) and a 70-fold concentration of the medium did not change this result (data not shown). This was not the case with *A. saitoi*, which secreted easily detectable amounts of mannosidase in medium containing yeast extract or glucose as carbon source. Hence, intracellular mannosidase is suggested based on characterization of 1,2-α-D-mannosidase-trimmed *N*-glycans (Maras et al., 1997a), which were synthesized by *T. reesei* RUTC 30 in a medium where no detectable extracellular mannosidase activity was measured. These observations might be explained by the fact that the peptide recognized as a signal peptide in *P. pastoris* does not function as such in the natural host *T. reesei*. Alternatively, another still unidentified 1,2-α-D-mannosidase may be present; perhaps differentially localized mannosidase variants are formed by alternative splicing (Francis et al., 1998). In order to explain these conflicting observations, the subcellular location of *T. reesei* 1,2-α-D-mannosidase is still under investigation.

In conclusion, a *T. reesei* 1,2-α-D-mannosidase has been cloned and characterized as being similar to previously cloned 1,2-α-D-mannosidases from *A. saitoi* and *P. citrinum*. The latter enzymes were found in the extracellular medium, and it is assumed that they are also involved in processing of protein-linked glycans. However, in the medium of *T. reesei* RUTC 30, no mannosidase activity was detected, whereas *N*-glycans synthesized under identical conditions were 1,2-α-D-mannosidase-trimming products. Hence, the above assumption that secreted 1,2-α-D-mannosidases are (the only ones) involved in processing *N*-glycans, becomes more questionable. Processing mechanisms to filamentous fungi may be more complex than assumed.

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